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Approved for Public Release; Distribution Unlimited**12b. DISTRIBUTION CODE****13. ABSTRACT (Maximum 200 Words)**

The hypothesis driving this project was validated since an oral DNA vaccine encoding murine VEGF-R2 (FLK-1) induced suppression of angiogenesis and ablation of tumor growth by triggering a specific CTL response specifically killing proliferating endothelial cells overexpressing FLK-1 in the tumor vasculature. Targeting of genetically stable endothelial cells rather than mutating, heterogeneous tumor cells was achieved in murine models of melanoma, colon- and lung carcinoma without impairment in fertility, hematopoiesis and neuromuscular performance and with only a slight decrease in wound healing. Novel FLK-1 DNA minigene vaccines were constructed and evaluated that encoded multiple, AAY-linked nonapeptides with H-2K^b/D^b anchors, including an ER signal peptide for optimal antigen processing and HIVtat for strong adjuvanticity. Another receptor tyrosine kinase, bFGF-R, overexpressed in proliferating endothelial cells in the prostate tumor vasculature proved an effective target for a DNA vaccine, inducing suppression of angiogenesis and inhibition of prostate tumor growth, especially when combined with secretory IL-15 which improved memory T Cell responses.

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INTRODUCTION

The overall objective is to induce suppression of tumor angiogenesis resulting in ablation of murine prostate tumors and their metastases in syngeneic C57Bl/6J mice. This will be done with oral DNA vaccines, carried by attenuated Salmonella typhimurium, encoding either the entire murine VEGF-R2 (FLK-1) gene or its different extracellular (ED), transmembrane (TD) or cytoplasmic (CD) functional domains; or FLK-1 nonapeptide minigenes with H2D^b or H2K^b anchors in combination with CD40 ligand trimer (CD40LT). Emphasis is placed on demonstrating optimal activation of T- and dendritic cells mediating effective killing of proliferating endothelial cells overexpressing FLK-1 in the prostate tumor vasculature that leads to suppression of angiogenesis and subsequent tumor ablation. This will be achieved by optimizing FLK-1 antigen processing in the proteasome and presentation by dendritic cells of MHC-class I antigen/FLK-1 peptide complexes to T cell receptors effectively stimulated by CD40LT, polyubiquitination of whole FLK-1 genes or, alternatively, insertion of an ER signal peptide, HIVtat and AAY linkers of FLK-1 nonapeptide minigene vaccines.

Our DNA vaccine approach is both novel and unique since it changed the target from genetically unstable tumor cells with heterogenous MHC antigen expression to genetically stable proliferating endothelial cells in the tumor vasculature which stably express MHC antigens essential for effective T cell-mediated immune responses. Our initial paper published on this subject in NATURE MEDICINE (Niethammer et al., 8:1369-1375, 2002) received world wide attention and extensive press coverage as well as many requests for our FLK-1 plasmid from research investigators throughout the world.

BODY

Task 1: (Months 1-15) as outlined in the initial grant proposal and in the currently revised SOW was mostly completed. The hypothesis driving this project was initially validated since proof of concept was established for the DNA vaccine encoding FLK-1 to induce suppression of angiogenesis in the tumor vasculature leading to T cell-mediated killing of proliferating endothelial cells that resulted in effective ablation of tumor growth and metastases in mouse models of melanoma, colon- and lung carcinoma (see NATURE MEDICINE paper in Appendix). Furthermore, effective metastatic prostate cancer models were established and standardized in syngeneic C57Bl/6J mice in both prophylactic and therapeutic settings. Additionally, a dual-function DNA vaccine encoding the entire genes for FLK-1 and CD40LT was constructed and validated as were additional DNA vaccines encoding either the extracellular (ED) plus transmembrane (TD) or transmembrane (TD) plus cytoplasmic (CD) FLK-1 domains. Also, the efficiency of our attenuated bacterial carrier for intralymphoid delivery of DNA encoding FLK-1 was established by demonstrating efficient transfer of the expression vector encoding FLK-1 to Peyer's patches in the small intestine (Fig. 1, Appendix). (Months 5-8). The bioactivity of these multiple expression plasmids with emphasis on anti-angiogenic/anti-tumor activities is currently ongoing in a series of long-term in vivo experiments (Months 9-15).

Furthermore, work on Task 2 has been completed in part by the construction and validation of FLK-1 nonapeptide minigene vaccines with H2D^b or H2K^b anchors (Fig 2, Appendix), including H2D^b or H2K^b binding motif predictions and scores (Table 1, Appendix). Immunization protocols for these minigene vaccines were established and

standardized (Fig. 3, Appendix), their CTL-mediated protective immunity against prostate cancer cells was demonstrated in vitro (Fig.4, Appendix) and their in vivo anti-tumor activity shown with the minigene vaccine expressing H2D^b anchors residues being most effective in both, therapeutic (Fig. 5A, Appendix) and prophylactic settings (Fig. 5B, Appendix). In addition, novel DNA vaccines were constructed encoding polyubiquitin with either the extracellular and transmembrane domains (ED/TD) or the transmembrane and cytoplasmic domains (TD/CD) of FLK-1. The constructs made are depicted, including their validation by Western blots (Fig. 6, Appendix). Importantly, during our first year's research effort, we also obtained new findings which will effectively strengthen our project. Specifically, we constructed and evaluated a novel DNA vaccine encoding another receptor tyrosine kinase, basic fibroblast growth factor receptor (bFGF-R), which is also overexpressed on proliferating endothelial cells in the prostate tumor vasculature. Initial results already demonstrated that this DNA vaccine is capable of ablating prostate cancer growth in a prophylactic setting, especially when also encoding secretory interleukin-15 (IL-15) known to improve memory T cell responses (Table 2, Appendix).

KEY RESEARCH ACCOMPLISHMENTS

- 1) The hypothesis driving this research project was validated since an oral DNA vaccine encoding murine VEGF-R2 (FLK-1) induced suppression of tumor angiogenesis and ablation of tumor growth via a specifically induced T cell response killing proliferating endothelial cells overexpressing FLK-1 in the tumor vasculature.
- 2) This FLK-1-based DNA vaccine proved effective in syngeneic models of murine melanoma, colon- and lung carcinoma.
- 3) The FLK-1-based vaccine could induce markedly reduced dissemination of spontaneous and experimental metastases of lung carcinoma.
- 4) The vaccine proved effective in both prophylactic and therapeutic settings and induced a long-lasting memory T cell response.
- 5) Suppression of tumor angiogenesis was achieved without impairment in fertility, hematopoiesis, neuromuscular performance and only a slight delay in wound healing.
- 6) Novel constructs of FLK-1 nonapeptide minigene-based DNA vaccines were completed with either H2D^b or H2K^b anchors, including an ER signaling peptide for improved antigen processing and HIVtat for adjuvanticity.
- 7) Anti-tumor efficacy of these minigene vaccines was demonstrated, with the vaccine encoding H2D^b anchors being most effective, both in inducing tumor-specific CTLs and in suppressing prostate tumor growth in prophylactic as well as therapeutic settings.
- 8) New vaccine constructs were completed encoding either the extracellular/transmembrane domains or the transmembrane/cytoplasmic domains of FLK-1 and their correct structures were validated by nucleotide sequencing and Western Blotting.

9) An orthotopic tumor model as well as an experimental metastasis model of prostate carcinoma were established and standardized.

10) A novel DNA vaccine encoding basic fibroblast growth factor receptor (bFGF-R) was constructed which also encoded secretory IL-15. This vaccine effectively suppressed prostate cancer growth in a prophylactic setting. This DNA vaccine which targets a receptor tyrosine kinase other than FLK-1, overexpressed on proliferating angiogenic endothelial cells, could possibly be used in combination with our FLK-1-based DNA vaccine for optimal anti-prostate tumor efficacy.

REPORTABLE OUTCOME:

Reportable outcomes validating the hypothesis driving this project were reported in our NATURE MEDICINE paper, published in December 2002 (see Appendix). Suppression of angiogenesis and ablation of tumor growth was achieved with the FLK-1-based DNA vaccine in syngeneic models of melanoma, colon- and lung carcinoma. Funding from this award also stimulated a new research finding as a novel DNA vaccine encoding endoglin, which is part of the TGF- β 1/ β 3 receptor complex, effectively induced T cell-mediated killing of proliferating endothelial cells in the prostate carcinoma vasculature. This led to effective tumor protective immunity against prostate carcinoma cell challenges and resulted in greater than 10-fold reduction in tumor mass versus empty vector and PBS controls.

CONCLUSIONS:

A key finding of the first year of this project was the validation of the hypothesis driving this prostate cancer therapy project as demonstrated in our NATURE MEDICINE paper (December, 2002). The effective anti-tumor activity demonstrated by one of our FLK-1 minigene vaccines and the development of a bFGF-R-based DNA vaccine which proved effective in ablating prostate cancer growth in prophylactic and therapeutic settings considerably strengthened this entire project. The novel and effective endoglin-based DNA vaccine added further strength.

APPENDICES:

Fig. 1, p12;

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Assurance/Certification/Declaration, p19;

Publication: Niethammer, A.G., Xiang, R., Becker, J.C., Wodrich, H., Pertly, U., Karsten, G., Eliceiri, B.P. and Reisfeld, R.A. A DNA vaccine against vascular endothelial growth factor receptor 2 prevents effective angiogenesis and inhibits tumor growth. Nat. Med., 8:1369-1375, 2002.

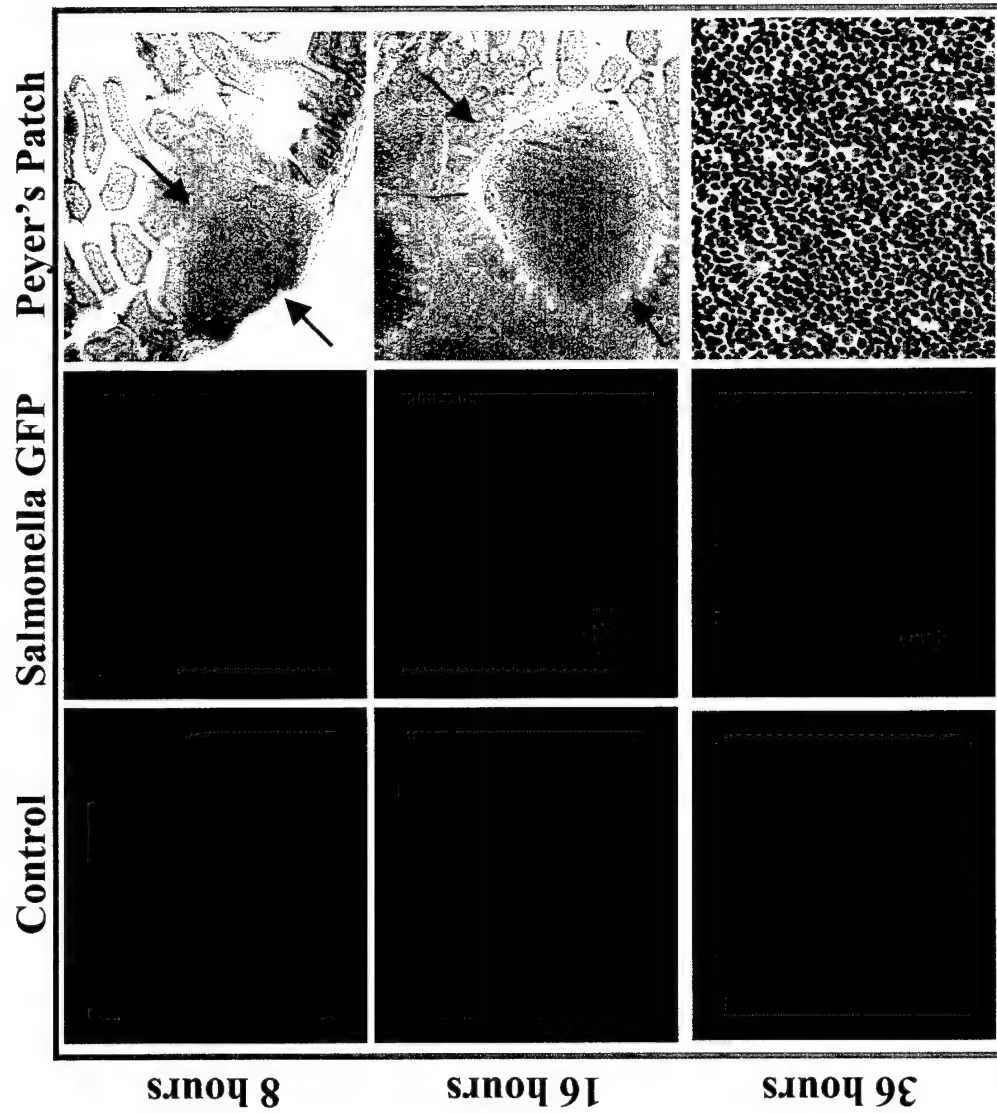


Fig. 1 Evidence for EGFP expression and transfer of the expression vectors from the double attenuated salmonella to mouse Peyer's Patches.

Table 1: Predictions for H-2D^b/H-2K^b binding motifs

Nona-Motif	Rank	Position	Motif	Binding Score
H-2D ^b	1	400	VILTNPISM	660.0
	4	1210	FHYDNTAGI	220.0
	15	94	RVVGNDTGA	36.0
H-2K ^b	1	1129	TTPEMYQTM	86.4
	9	771	VIAMFFWLL	12.0
	18	54	RGQRDLDWL	4.75

Fig.2 Schematic representation of Flk-1 minigene-based DNA vaccine expression constructs:**H-2D^b construct:****H-2K^b construct:**

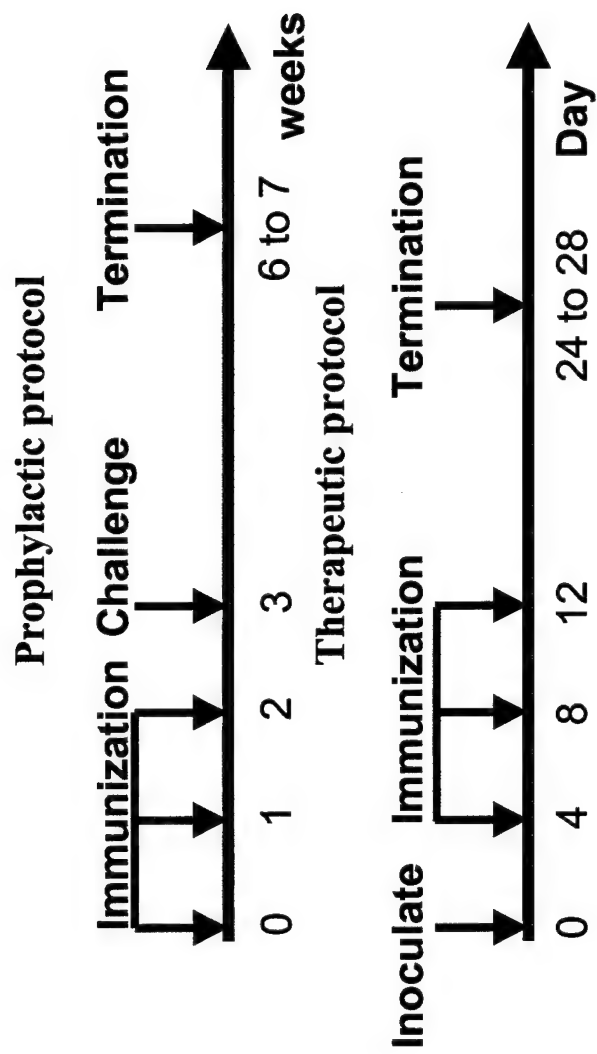


Fig.3 Immunization Protocols for DNA Minigene Vaccines

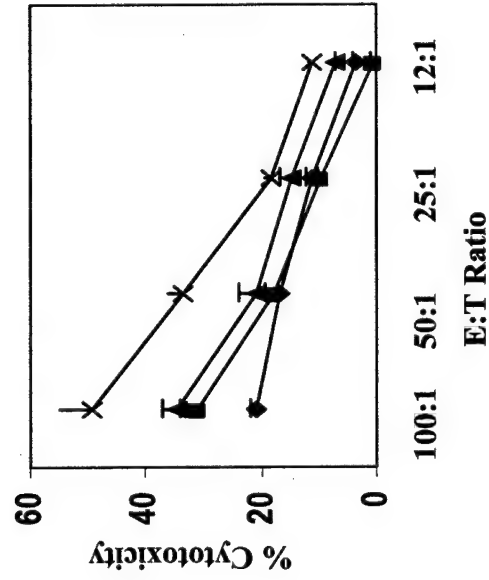
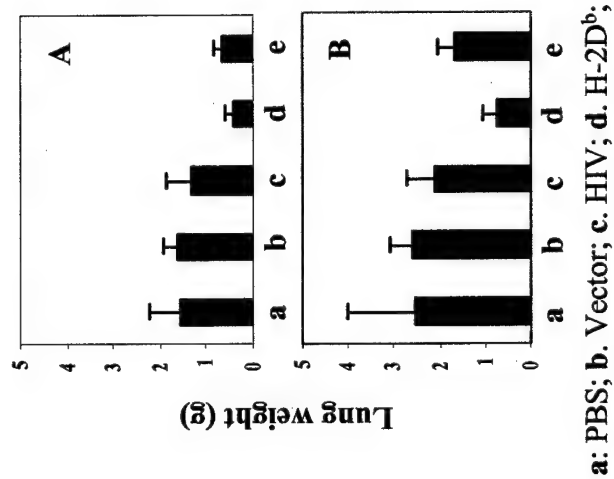


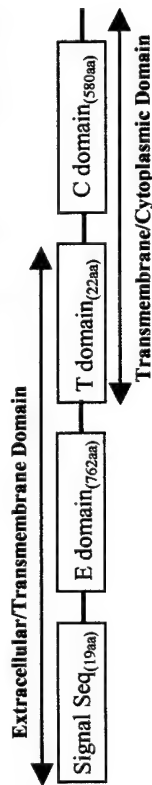
Fig. 4 CTL mediated tumor protective immunity induced by Flk-1 minigene-based DNA vaccine against prostate cancer. (◆) : vector; (■): HIVtag; (x): H-2D^b; (▲): H-2K^b;



a: PBS; b. Vector; c. HIV; d. H-2D^b; e. H-2K^b

Fig. 5 Growth suppression of prostate tumors by an oral Flk-1 minigene-based DNA vaccine in a syngeneic C57BL/6J mouse model

A. Basic construct and predicted amino acid sequence of Flk-1 cDNA
Encoding extracellular, transmembrane and cytoplasmic domains



B. Expression plasmids for oral vaccination

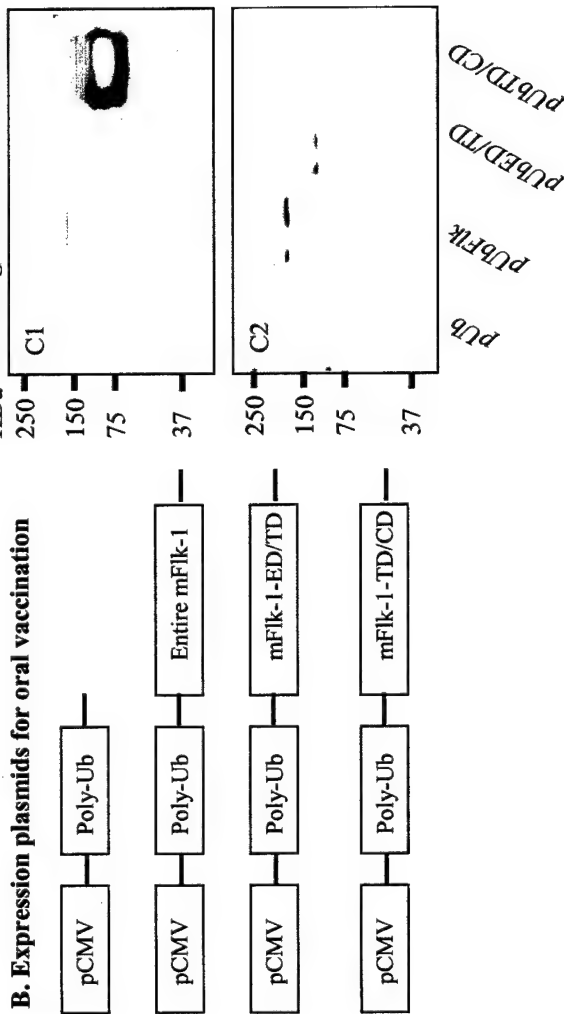


Fig6. Schematic diagram of Flk-1 gene and protein expression. In Fig.1A, the entire Flk-1 gene is shown including each of its bio-functional domain. DNAs encoding the whole Flk-1 gene or different domains were inserted into the pBud vector at the C-terminal of poly-ubiquitin for properly antigen procession and presentation (Fig1. B.). The constructs were verified by nucleotide sequencing and Western blots, shown in by Fig.1 C. The results of Western blot were confirmed by using either anti-Flk-1 C-terminal antibody(C1) or anti N-terminal antibody (C2).

Table 2. Effect of DNA vaccine encoding bFGF-R/secretory IL-15 on RM2 prostate cancer metastases

Experimental groups	Metastasis score
Empty vector	3, 3, 3, 3
hIL-15	1, 2, 3, 3
FGFR-long	0, 1, 1, 2
FGFR-long/hIL-15	0, 0, 0, 1

C57BL/6J mice were immunized with DNA vaccine encoding the long isoform of bFGF-r and 2 weeks later with the same vaccine additionally encoding secretory hIL-15. Two weeks thereafter these animals (n=4) were challenged by i.v. injection of 5×10^4 RM-2 prostate cancer cells and then evaluated 4 weeks later for lung metastases. Since these were fused, the lung surface covered by metastases was evaluated as follows: 0=no metastases; 1=<5%, 2=5-25%; 3=>50%.

ASSURANCE/ CERTIFICATION/DECLARATION

CARE AND USE OF ANIMALS

Animal Research Committee
The Scripps Research Institute

☒ Grant☐ Contract☐ Fellow☐ Other☐ New☐ Competing continuation☒ Noncompeting continuation☐ Supplemental

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3. PRINCIPAL INVESTIGATOR, PROGRAM DIRECTOR, OR FELLOW

POLICY:

Any research activity involving animals undertaken at The Scripps Research Institute (TSRI) must be reviewed and approved by our Institutional Animal Care and Use Committee (IACUC), the Animal Research Committee (ARC), in accordance with Public Health Policy regarding the care and use of laboratory animals. Applicants are required to submit to all granting agencies certification of ARC approval of compliance with the Guide for the Care and Use of Laboratory Animals, the American Association for the Accreditation of Laboratory Animal Care (AAALAC) standards, and the regulations set forth in the Animals Welfare Act (P.L. 89-544, as amended by P.L. 91-579 and P.L. 94-279) and other applicable federal, state and local laws, regulations and policies. The Scripps Research Institute currently has an assurance (A3194-01) of compliance on file with the U.S. Department of Health and Human Services (HHS) which covers the Institution's responsibility to monitor animal welfare. In the event that an application receives a pending approval from the ARC covering the proposed activity, certification of the ARC review and approval must be submitted to the granting agency within 60 days of filing the grant application.

4. HHS ASSURANCE STATUS

- ☒ The Scripps Research Institute has an approved assurance of compliance on file with HHS (A3194-01) which covers the care and use of laboratory animals and furthermore is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

5. CERTIFICATION OF ARC REVIEW

- ☐ The applicant has sought approval of the portions of this research activity which include laboratory animals from the ARC of The Scripps Research Institute in accordance with HHS recommendations and AAALAC standards.

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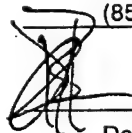
Date of ARC review and approval. (If approval is pending, write "pending". Follow-up certification is required within 60 days of filing grant application.)

- ☐ This activity contains multiple projects, some of which involve other institution(s) that have not been reviewed by the ARC. The ARC has granted approval on the condition that all projects involving laboratory animals will be reviewed and approved by an accredited Institutional Animal Care and Use Committee (IACUC) before they are initiated and that appropriate certification will be submitted by the responsible institution.

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A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth

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Tumor cells are elusive targets for immunotherapy due to their heterogeneity and genetic instability. Here we describe a novel, oral DNA vaccine that targets stable, proliferating endothelial cells in the tumor vasculature rather than tumor cells. Targeting occurs through upregulated vascular-endothelial growth factor receptor 2 (FLK-1) of proliferating endothelial cells in the tumor vasculature. This vaccine effectively protected mice from lethal challenges with melanoma, colon carcinoma and lung carcinoma cells and reduced growth of established metastases in a therapeutic setting. CTL-mediated killing of endothelial cells indicated breaking of peripheral immune tolerance against this self antigen, resulting in markedly reduced dissemination of spontaneous and experimental pulmonary metastases. Angiogenesis in the tumor vasculature was suppressed without impairment of fertility, neuromuscular performance or hematopoiesis, albeit with a slight delay in wound healing. Our strategy circumvents problems in targeting of genetically unstable tumor cells. This approach may provide a new strategy for the rational design of cancer therapies.

The inhibition of tumor growth by attacking the tumor's vascular supply offers a primary target for anti-angiogenic intervention. This approach, pioneered by Folkman and colleagues^{1–5}, is attractive for several reasons. First, the inhibition of tumor-associated angiogenesis is a physiological host mechanism and should not lead to the development of resistance. Second, each tumor capillary has the potential to supply hundreds of tumor cells, so that targeting the tumor vasculature actually potentiates the antitumor effect. Third, direct contact of the vasculature with the circulation leads to efficient access of therapeutic agents⁶.

Extensive studies by many investigators established that angiogenesis has a central role in the invasion, growth and metastasis of solid tumors^{2,7–9}. In fact, angiogenesis is a rate-limiting step in the development of tumors since tumor growth is generally limited to 1–2 mm³ in the absence of a blood supply^{6,10}. Beyond this minimum size, tumors often become necrotic and apoptotic under such circumstances¹¹.

Because tumor cells frequently mutate in response to therapy and also downregulate major histocompatibility (MHC) antigens required for T cell-mediated antitumor responses^{12,13}, efforts have been made to eradicate tumors by therapies directed against the tumor microenvironment. One such report links calreticulin with a model viral tumor antigen, thus combining antitumor therapy with anti-angiogenesis¹⁴. Yet another approach is the administration of xenogeneic endothelial cells as a vaccine that yielded anti-angiogenic effects¹⁵. This approach differs from those of other investigators applying specific chemical or biological inhibitors of angiogenesis, which often require their constant administration at relatively high dose levels¹⁶.

A more molecularly-defined alternative to xenoimmunization is offered by receptor tyrosine kinases (RTKs) and their growth-factor ligands required for tumor growth. Among these receptors, the vascular endothelial growth factor receptor 2 (VEGFR2, also known as FLK-1) that binds the five isoforms of murine VEGF has a more restricted expression on endothelial cells and is upregulated once these cells proliferate during angiogenesis in the tumor vasculature. FLK-1 is strongly implicated as a therapeutic target, as it is necessary for tumor angiogenesis and has an important role in tumor growth, invasion and metastasis^{7,8,17–24}. In fact, several approaches have been used to block FLK-1, including dominant-negative receptor mutants, germ-line disruption of VEGFR genes, monoclonal antibodies against VEGF and a series of synthetic RTK inhibitors^{24,25}.

Here we describe a novel strategy for achieving an antitumor immune response with a FLK-1-based DNA vaccine. Our vaccine causes the collapse of tumor vessels by evoking a T cell-mediated immune response against proliferating endothelial cells overexpressing this growth-factor receptor in the tumor vasculature.

A FLK-1 based DNA vaccine inhibits tumor growth

We tested our hypothesis by demonstrating that an effective antitumor immune response was induced against subcutaneous tumors by an orally administered DNA vaccine encoding murine FLK-1 carried by attenuated *Salmonella typhimurium*. To this end, we constructed the vector pcDNA3.1-FLK1 (Fig. 1a). Protein expression of FLK-1 was demonstrated by western blot-

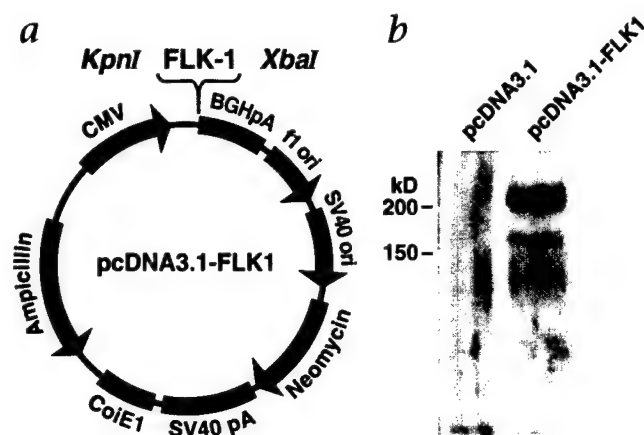


Fig. 1 Construction and functionality of expression vector. **a**, The DNA encoding the entire murine Flk-1 gene was inserted into the pcDNA3.1 vector between the restriction sites *KpnI* (5') and *XbaI* (3'). **b**, This construct was verified by nucleotide sequencing and protein expression by western blots after transient transfection into COS-7 cells. The protein appears in the lysate in its glycosylated form at 220 kD and to a lesser extent in its unglycosylated form at approximately 150 kD.

ting of transfected COS-7 cells (Fig. 1b). We established the efficacy of gene transfer from attenuated *S. typhimurium* into Peyer's patches by GFP expression in the cells derived from Peyer's patches at different time points after oral administration of mice (data not shown).

Marked inhibition of subcutaneous (s.c.) tumor growth was observed in C57BL/6J mice challenged two weeks after the third vaccination with pcDNA3.1-FLK1 by s.c. injection of either B16G3.26 murine melanoma cells or D121 non-small cell Lewis lung carcinoma cells (Figs. 2a and b). In contrast, animals vaccinated with only the empty vector

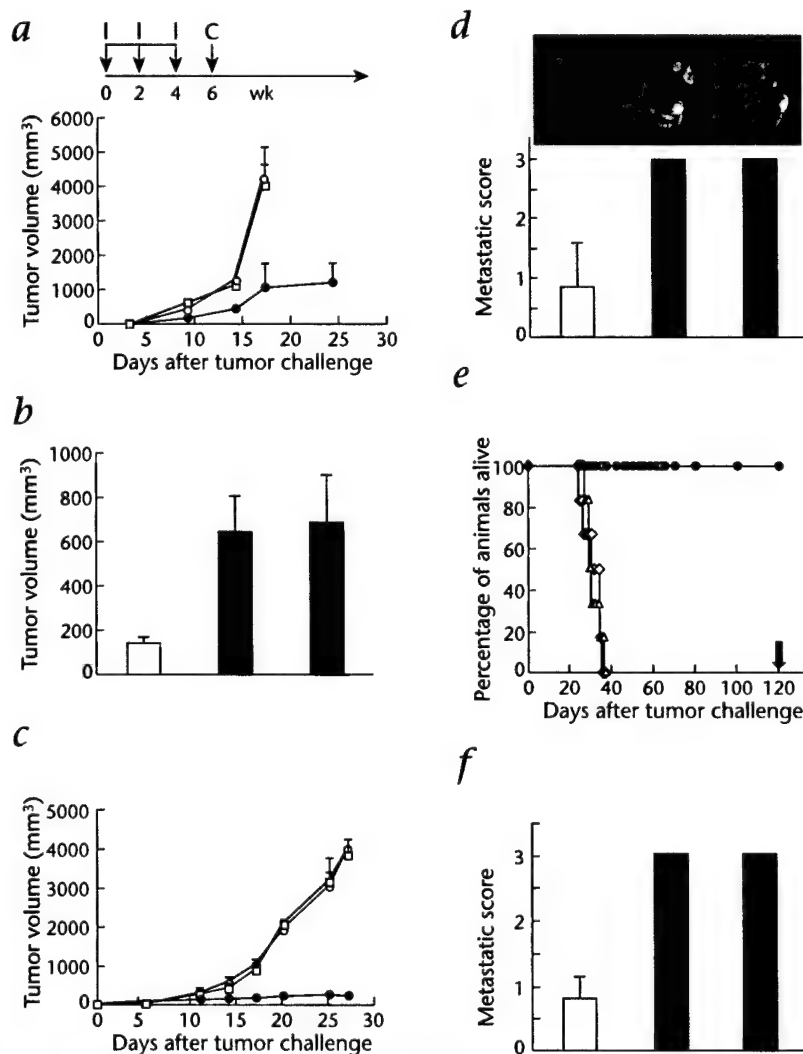
pcDNA3.1, carried by the attenuated bacteria, revealed uniformly rapid s.c. tumor growth.

Prolonged antitumor effects were demonstrated since C57BL/6J mice challenged s.c. with MC-38 colon carcinoma cells 10 months after their last vaccination revealed a marked decrease in tumor growth in all experimental animals compared with controls (Fig. 2c).

Protection against spontaneous pulmonary metastases

We noted a marked reduction in dissemination of spontaneous pulmonary metastases in all experimental animals following three immunizations with the FLK-1-based DNA vaccine. This became evident 30 days after surgical excision of the s.c. primary Lewis lung carcinoma tumors, as confirmed by visual examination of the lungs of these animals, which established their metastatic score (Fig. 2d), as well as by histological analyses (data not shown).

Fig. 2 Effect of the FLK-1 based DNA vaccine on tumor growth. **a**, 2 wk after the last vaccination, mice were challenged (c) with a lethal dose of B16 melanoma cells. The average tumor growth of 8 mice is depicted. ●, immunization (I) with the vector encoding FLK-1; ○, control vector; □, PBS. $P < 0.01$. **b**, C57BL/6J mice ($n = 8$) were challenged s.c. with a lethal dose of 1×10^5 D121 Lewis lung carcinoma cells (□). Bar graphs indicate average tumor volume after 2 wk before tumor removal in comparison with mice that received only the control vector (■) or PBS (■). Error bars indicate s.d. **c**, Long-term effect of the pcDNA3.1-FLK1 vaccination was tested by challenging C57BL/6J mice ($n = 8$) with MC-38 colon carcinoma s.c. 10 months after the third immunization. Symbols are as in **e**. **d**, Representative lung specimens of mice challenged with D121 Lewis lung carcinoma cells 4 weeks after removal of the subcutaneous primary tumors. Bars are as in **b**. **e**, Lifespan of BALB/c mice after i.v. challenge with CT-26 colon carcinoma cells. The lifespan of groups of mice ($n = 6$) are shown following tumor cell challenge after repeated vaccinations. Death occurred in control groups due to extensive metastatic dissemination throughout the lung. 5 of the surviving mice were rechallenged (black arrow) to test for possible resistance. ●, vaccine; ◇, empty vector control; △, PBS. **f**, Inhibition of tumor growth in a therapeutic setting. BALB/c mice ($n = 5$) were challenged i.v. with CT-26 colon carcinoma, and 10 d later 1 dose of the vaccine was applied. Experimental groups were scored 28 d after challenge. Bars are as in **b**.



Vaccination prolongs the lifespan of mice

We found a fourfold increase in lifespan of BALB/c mice ($n = 6$), vaccinated as described above, and challenged two weeks later by intravenous (i.v.) injection of a lethal dose of autologous CT-26 colon carcinoma cells (Fig. 2e). Possible resistance against our therapy was ruled out by rechallenging survivors ($n = 5$) 120 days after their first tumor-cell challenge and collecting their lungs 30 days later. Four mice did not reveal any tumors, whereas the one remaining animal had less than 10% of its lung surface covered by metastases (data not shown).

Vaccination reduces growth of established metastases

We established that our FLK-1-based DNA vaccine is also effective in a therapeutic setting. This was shown by i.v. injection of BALB/c mice ($n = 5$) with CT-26 colon carcinoma cells and vaccination of these mice 10 days thereafter with pcDNA3.1-FLK1 when they had fully established pulmonary metastases. All such treated mice survived and showed only few small lung foci, whereas all control animals treated with the empty vector or PBS began to die 28 days after tumor cell challenge (Fig. 2f).

CD8⁺ T cells are responsible for the antitumor response

There was a marked increase in T-cell activation markers in splenocytes from successfully vaccinated C57BL/6J mice after a 12-hour incubation with B16G3.26 melanoma cells that had

been stably transduced to express murine FLK-1. This included increased expression of CD25, the high-affinity interleukin-2 (IL-2)-receptor α chain, CD69 an early T-cell activation antigen and LFA-2 (CD2) a lymphocyte function-associated antigen (Fig. 3a). This upregulation was clearly evident when compared with CD8⁺ T cells from mice vaccinated with pcDNA3.1-FLK1 but incubated with wild-type B16G3.26 melanoma cells ($P \leq 0.05$). Specific recognition of FLK-1 was indicated as no increase in expression was noted following co-incubation of cells expressing FLK-1 with splenocytes from C57BL/6J mice vaccinated with the empty vector. No such upregulation could be observed for CD4⁺ T cells (data not shown).

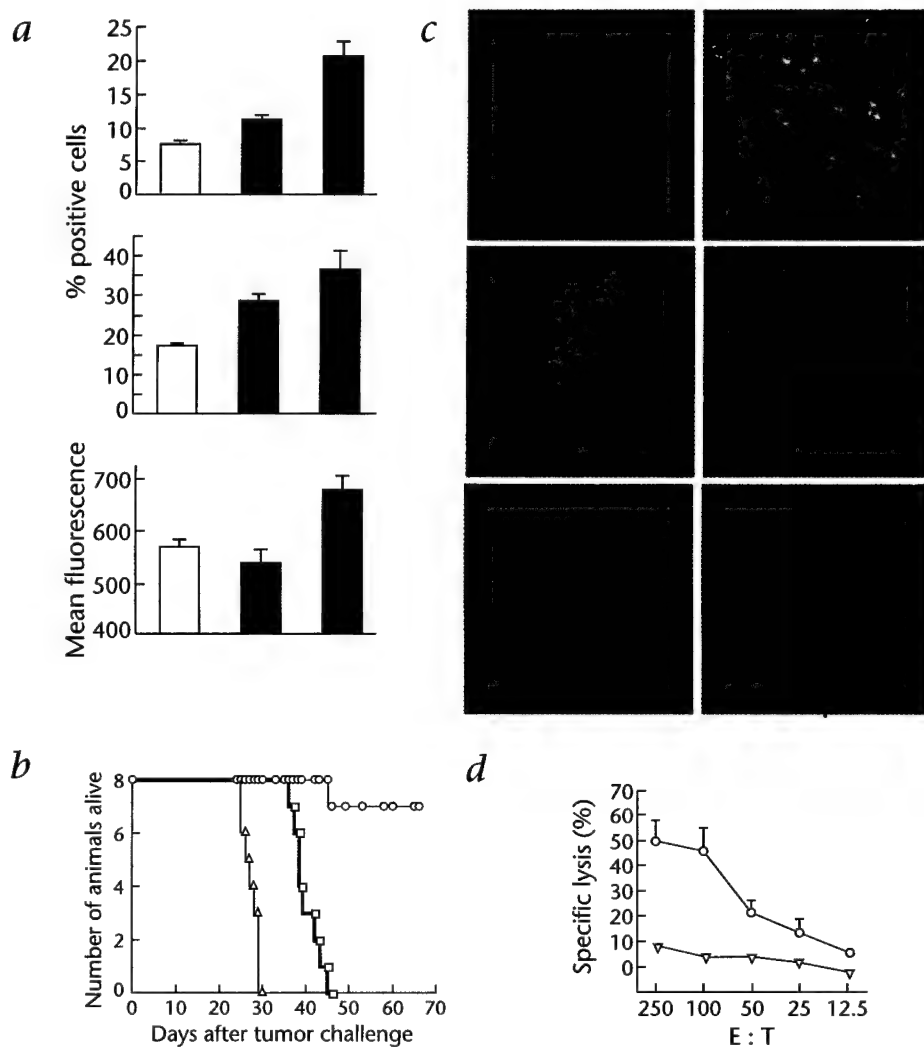
We demonstrated the involvement of CD8⁺ T cells in the antitumor immune response given that *in vivo* depletion of CD8⁺ T cells—before i.v. challenge of vaccinated mice with CT-26 tumor cells—resulted in the complete abrogation or severe impairment of the antitumor response. In fact, mice depleted of CD8⁺ T cells died within 45 days after tumor-cell challenge due to extensive growth and dissemination of pulmonary metastases (Fig. 3b). However, *in vivo* depletion of CD4⁺ T cells did not decrease the effectiveness of our vaccine (data not shown).

Cytotoxic T cells associate with tumor endothelium

To reveal the localization of CD8⁺ T cells to their target site, we stained these cells with fluorescein isothiocyanate (FITC) and

Fig. 3 Involvement of CD8⁺ T cells.

a, Activation of CD8⁺ T cells after *in vitro* co-incubation with cells expressing FLK-1. Shown is the increase in percentage of CD8⁺CD25⁺ (top) and CD8⁺CD69⁺ (middle) T cells, and increase in expression of CD2 on CD8⁺ T cells (bottom) isolated from splenocytes of mice vaccinated with the vector pcDNA 3.1-FLK1 after cocultivation with cells expressing FLK-1 (■), or the identical cells lacking FLK-1 (□). A further control included CD8⁺ T cells from mice immunized with the empty control vector and cocultivated with the B16 melanoma cell line expressing FLK-1 (□). Error bars indicate s.d. **b**, Effect of CD8⁺ T-cell depletion on lifespan. □, CD8⁺ T-cell depletion during effector phase after vaccination with pcDNA3.1-FLK1; ○, no CD8⁺ T-cell depletion of vaccinated mice; △, empty control vector. **c**, Immunohistochemical analysis of CD8⁺ T cells (FITC) and endothelial cells (rhodamine). Upper panels depict vascularized areas of CT-26 pulmonary metastases 4 mo after challenge and immunization with pcDNA3.1-FLK1. Left, $\times 20$ magnification, $530 \times 530 \mu\text{m}$; right, $\times 40$ magnification, $265 \times 265 \mu\text{m}$. Middle panels show Matrigel specimens after bFGF-induced vessel growth and prior immunizations with pcDNA3.1-FLK1 (left) and empty vector (right). Both panels, $\times 20$ magnification. Lower panels reveal non-vascularized areas of tumor tissue (left) and adjacent skin tissue (right). Both panels, $\times 20$ magnification. **d**, Specific lysis of CT-26-FLK-1 cells by CD8⁺ T cells from mice vaccinated with pcDNA3.1-FLK-1 (○) compared with CD8⁺ T cells from control mice treated with empty vector (▽).



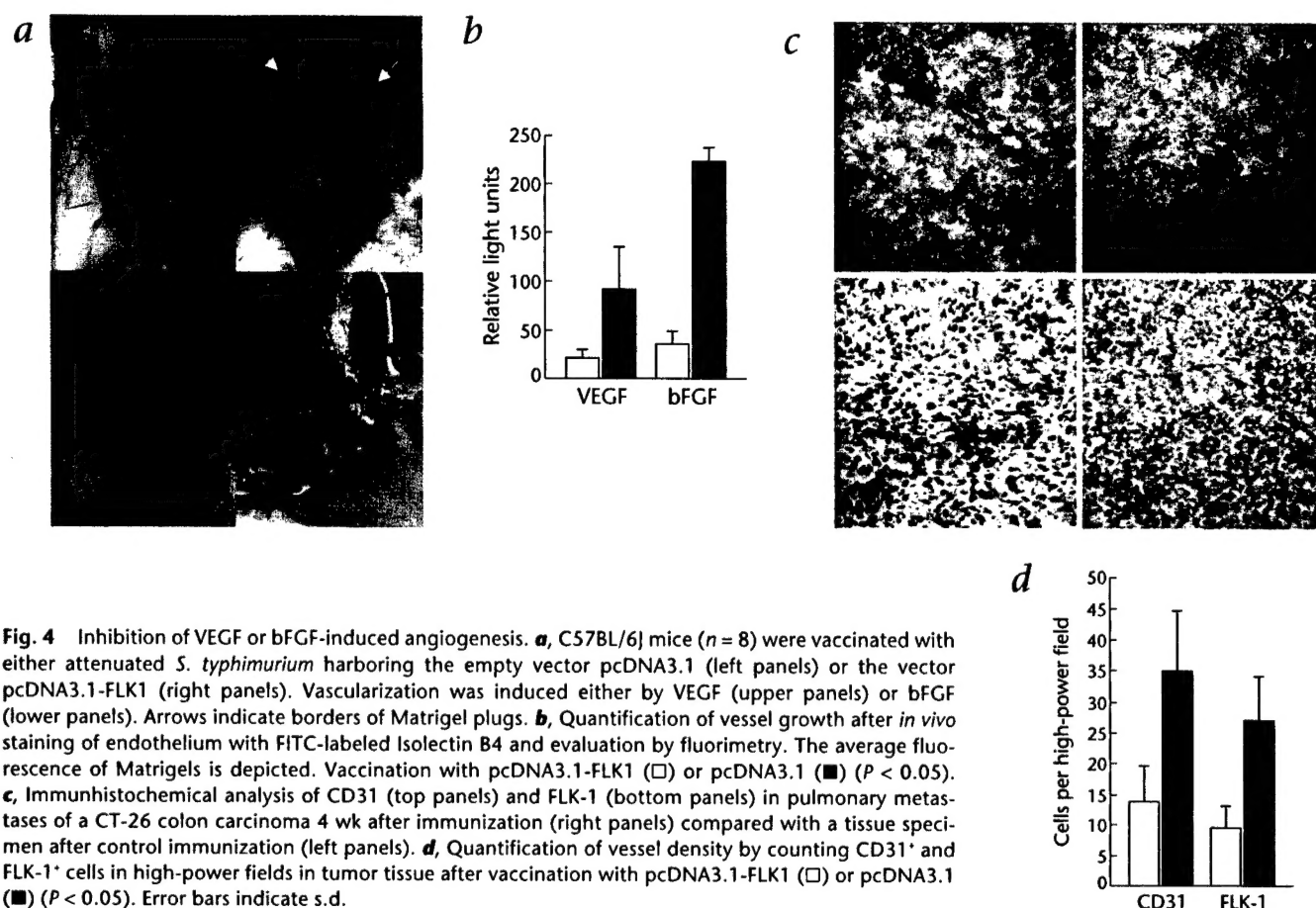


Fig. 4 Inhibition of VEGF or bFGF-induced angiogenesis. **a**, C57BL/6J mice ($n = 8$) were vaccinated with either attenuated *S. typhimurium* harboring the empty vector pcDNA3.1 (left panels) or the vector pcDNA3.1-FLK1 (right panels). Vascularization was induced either by VEGF (upper panels) or bFGF (lower panels). Arrows indicate borders of Matrigel plugs. **b**, Quantification of vessel growth after *in vivo* staining of endothelium with FITC-labeled Isolectin B4 and evaluation by fluorimetry. The average fluorescence of Matrigels is depicted. Vaccination with pcDNA3.1-FLK1 (\square) or pcDNA3.1 (\blacksquare) ($P < 0.05$). **c**, Immunohistochemical analysis of CD31 (top panels) and FLK-1 (bottom panels) in pulmonary metastases of a CT-26 colon carcinoma 4 wk after immunization (right panels) compared with a tissue specimen after control immunization (left panels). **d**, Quantification of vessel density by counting CD31⁺ and FLK-1⁺ cells in high-power fields in tumor tissue after vaccination with pcDNA3.1-FLK1 (\square) or pcDNA3.1 (\blacksquare) ($P < 0.05$). Error bars indicate s.d.

marked endothelial cells with rhodamine-conjugated antibody against CD31. Microscopic evaluation revealed an association of CD8⁺ T cells with vessel structures throughout the tumor tissue or Matrigel sections of animals immunized with pcDNA3.1-FLK1. Almost no CD8⁺ T cells were observed within non-vascularized, viable areas of tumor tissues even four months after tumor-cell challenge, nor were they associated with vessels in somatic tissues. Control vaccination did not induce any infiltration of cytotoxic T cells into tumor tissue or Matrigel (Fig. 3c).

Vaccination against FLK-1 induces T cell-mediated lysis

We demonstrated antigen-specific cytotoxicity against CT-26-FLK-1 cells with a standard 4-hour ⁵¹Cr-release assay using splenocytes from BALB/c mice immunized against FLK-1 and challenged with CT-26 colon carcinoma cells. Immunizations with the vector encoding FLK-1 led to significant lysis of target cells by effector cells, in contrast to control immunizations (Fig. 3d). However, neither vaccination was effective in evoking any noticeable cytotoxicity against wild-type CT-26 cells not expressing FLK-1, thus excluding direct lysis of tumor cells (data not shown).

Reduction of neovascularization

We demonstrated distinct anti-angiogenic effects, independent of tumor cells, induced by the FLK-1-based DNA vaccine in a Matrigel assay. Differences were visible macroscopically, as shown in representative examples of Matrigel plugs removed six days after their installment (Fig. 4a). This was also evident from the extent of vascularization evaluated by relative fluorescence after *in*

vivo staining of endothelium with FITC-conjugated lectin. There was a decrease in VEGF- or bFGF-induced neovascularization only after vaccination with the vector encoding FLK-1 but not with the empty vector (Fig. 4b). Immunohistochemical staining further revealed a decrease in vessel density among pulmonary metastases of CT-26 colon carcinoma after vaccination with pcDNA3.1-FLK1 as compared with tissue derived from control mice (Fig. 4c). Evaluation of high-power fields demonstrated decreased vessel density induced by the FLK-1-based vaccine (Fig. 4d).

Wound healing is delayed after vaccination

We noticed a measurable prolongation in the time required to completely close a total of 24 circular wounds inflicted on the backs of 6 mice immunized with the FLK-1-based vaccine versus that of mice immunized with the control vector (14.75 days, s.d. 1.5 versus 13.3 days, s.d. 1.6; $P < 0.01$). This was accompanied by macroscopically visible swelling and inflammation in 11 of 24 versus 4 of 24 cases among controls (Fig. 5a and b).

Further experiments revealed no impact on fertility of mice based on the time elapsed from start of cohabitation until parturition nor on the number of pups born (Fig. 5c and d). All females of each experimental group gave birth. Neuromuscular performance as determined by both the wire test and footprint test, as well as by body weight, overall behavior and balancing tests, did not demonstrate any impairment attributed to vaccination (data not shown).

The occurrence of common, FLK-1-positive progenitor cells for both endothelial cells and hematopoietic cells led us to evaluate peripheral blood samples of C57BL/6J and BALB/c

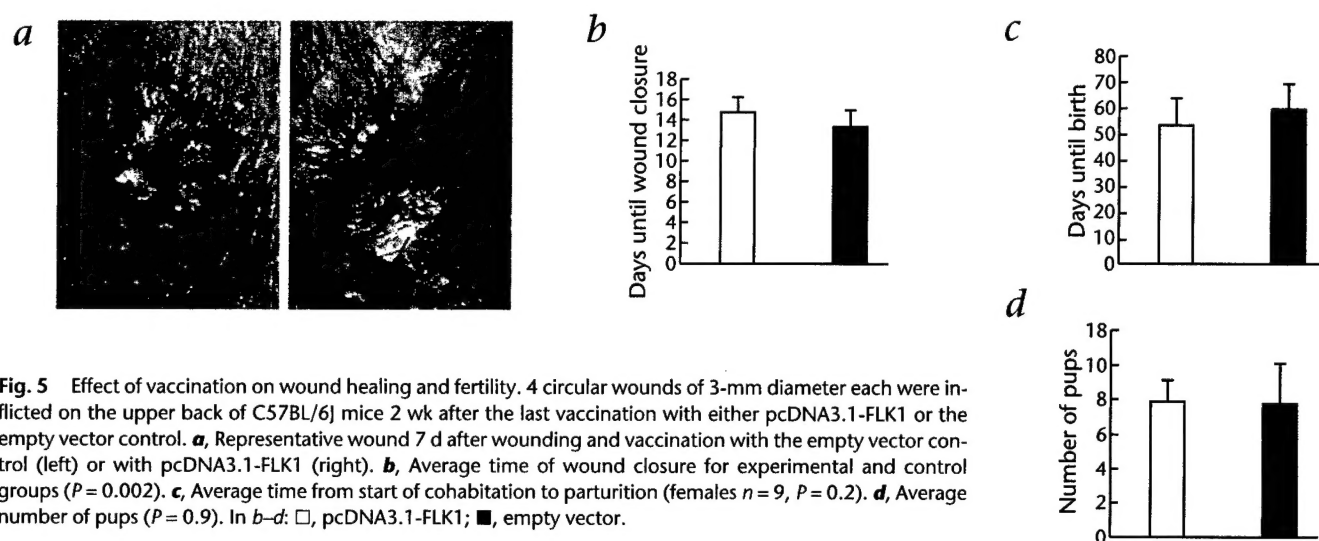


Fig. 5 Effect of vaccination on wound healing and fertility. 4 circular wounds of 3-mm diameter each were inflicted on the upper back of C57BL/6J mice 2 wk after the last vaccination with either pcDNA3.1-FLK1 or the empty vector control. **a**, Representative wound 7 d after wounding and vaccination with the empty vector control (left) or with pcDNA3.1-FLK1 (right). **b**, Average time of wound closure for experimental and control groups ($P = 0.002$). **c**, Average time from start of cohabitation to parturition (females $n = 9$, $P = 0.2$). **d**, Average number of pups ($P = 0.9$). In **b–d**: □, pcDNA3.1-FLK1; ■, empty vector.

mice up to ten months after their last immunization. However, total blood counts and differentials did not indicate any decreased or compensating hematopoiesis (data not shown).

Discussion

We developed a novel strategy that might overcome problems of tumor-cell heterogeneity and peripheral tolerance to self-antigens encountered in tumor cell-directed immunotherapy. We accomplished this by exploiting the obvious advantages of anti-angiogenic therapies developed by many other investigators^{1–5}. In fact, there are several advantages of targeting CD8⁺ T cells to proliferating endothelial cells in the tumor vasculature rather than directly to tumor cells. First, endothelial cells are genetically stable and do not downregulate MHC-class I and II antigens—an event that frequently occurs in solid tumors and severely impairs T cell-mediated antitumor responses¹². In addition, immune suppression triggered by tumor cells at the cellular level can also be avoided by this approach. Second, the therapeutic target is tumor-independent, thus killing of proliferating endothelial cells in the tumor microenvironment can be effective against a variety of malignancies. Furthermore, proliferating endothelial cells are readily available to lymphocytes in the bloodstream. Consequently, the target tissue can be reached unimpeded by anatomical barriers such as the blood-brain barrier or encapsulation of tumor tissues²⁶.

Our studies show that peripheral T-cell tolerance against the murine VEGFR2 (FLK-1) can be broken by an oral DNA vaccine encoding autologous FLK-1, delivered by an attenuated strain of *S. typhimurium*. We induced antitumor immune responses in mouse tumor models of non-small cell lung carcinoma, colon carcinomas and melanoma, both against primary tumors and their respective spontaneous and experimental pulmonary metastases. Our DNA-vaccine was also effective in a therapeutic setting of established lung metastases. Additionally, we observed effective, prolonged antitumor effects evident after challenge up to ten months after the last immunization. Involvement of cytotoxic T cells in these events was suggested by marked upregulation of T-cell activation markers CD2, CD25, CD69 on CD8⁺ T cells when co-incu-

bated with cells expressing FLK-1. There was no apparent up-regulation of these markers upon incubation with tumor cells. Furthermore, the effect of our vaccine was severely impaired in mice depleted *in vivo* of CD8⁺ T cells throughout the effector phase. Depletion of CD4⁺ cells was without effect. Importantly, angiogenesis was found to be effectively counteracted in a tumor cell-free, VEGF- or bFGF-induced Matrigel assay; both VEGF and bFGF upregulate the target antigen FLK-1 on endothelial cells²⁷. Furthermore, *in vitro* cytotoxicity occurred only against target cells transduced to express FLK-1, but not against the identical wild-type cell line. We eliminated cytotoxicity mediated by CD8⁺ T cells directly against tumor cells, as well as non-specific immune responses, as tumor protection was completely abrogated in mice that received attenuated *S. typhimurium* transformed with the empty vector lacking FLK-1.

We further demonstrated that fertility, neuromuscular performance and hematopoiesis of experimental mice remained unimpaired; anti-angiogenic effects induced by our DNA vaccine resulted in a slight but statistically significant delay in wound healing. However, the wounds of mice that were subjected to tumor excisions—including in some cases opening of the peritoneum—healed without any complications.

Together, our data show that the VEGFR2 is a suitable target for T cell-mediated immunotherapy within the tumor vasculature. Our findings may lead to a novel vaccine strategy for cancer therapy through the induction of an autoimmune response against self antigens expressed by proliferating endothelial cells. In fact, at least 46 transcripts are specifically elevated in the tumor-associated endothelium, thus providing a large array of potential candidates for this strategy²⁸. This includes integrins or additional growth factor receptors and their ligands such as basic fibroblast growth factor or angiopoietin, as well as other molecules involved in their downstream signaling events²⁸. It is also likely that DNA vaccines targeting proliferating endothelial cells could be used effectively in combination with specific inhibitors of angiogenesis, and chemotherapies or immunotherapies targeting the tumor cells themselves. Such combined approaches may ultimately lead to the rational design of novel and effective modalities for the treatment of cancer.

Methods

Animals, bacterial strains, and cell lines. Animal experiments were performed according to the National Institutes of Health *Guide for Care and Use of Experimental Animals* and approved by our Animal Care Committee (#ARC-43SEPT1). Attenuated *S. typhimurium* Aro/A (strain SL7207) was provided by B.A.D. Stocker. The D121 cell line was a gift from L. Eisenbach. Tumor tissues were screened for expression of FLK-1 by immunohistochemical staining and found to be negative; expression throughout the tumor-neovasculature was positive.

Construction of the expression vector encoding murine VEGFR-2 and transformation of *S. typhimurium*. DNA encoding murine VEGFR-2 (FLK-1) (provided by I. Lemischka) was cloned with the primers 3'-CCGGTA CCATGGAGAGCAAGGCCGCTG-5' and 5'-CCTCTAGACAGCAGCAC-CTCTCTC-3' and inserted into the pcDNA3.1 vector (Invitrogen, San Diego, California) between the restriction sites *Kpn*I and *Xba*I generating pcDNA3.1-FLK1. Bacteria were electroporated as described^{29,30}.

Oral immunization and tumor-cell challenge. Mice were immunized by oral gavage 3 times at 2-wk intervals with 100 μ l PBS containing 1×10^8 *S. typhimurium* transformed with pcDNA3.1-FLK1 or pcDNA3.1 as described³¹. C57BL/6J mice were challenged 2 wk later by s.c. injection of 1×10^5 B16G3.26 melanoma, MC-38 colon carcinoma or D121 lung carcinoma cells into the left front flank. Tumor volume was measured in 2 dimensions and calculated as follows: length/2 \times width². Tumors of mice injected with D121 cells were excised after 2 wk to allow spontaneous dissemination to the lung. Metastatic scores were evaluated 4 wk later by the percentage of lung surface covered by fused metastases: 0% = 0; <20% = 1; 20–50% = 2; and >50% = 3. We injected CT-26 murine colon carcinoma cells (5×10^4) i.v. into BALB/c mice inducing experimental pulmonary metastases 2 wk after the last immunization. We tested our treatment in a therapeutic setting by vaccinating animals 10 d after i.v. injection of CT-26 cells.

Activation of CD8⁺ T cells. We created the B16G3.26-FLK-1 melanoma and CT-26-FLK-1 colon carcinoma cell lines by retroviral transduction with FLK-1. One week after immunization, splenocytes were collected from C57BL/6J mice ($n = 4$), vaccinated with pcDNA3.1-FLK1 or the empty control vector. Cells were cocultured overnight with B16G3.26-FLK-1 or B16G3.26 tumor cells. Flow-cytometric analyses were performed using FITC-conjugated antibody to CD8 (#01044) in combination with PE-conjugated anti-mouse monoclonal antibodies to CD2 (#01175), CD25 (#01105A) or CD69 (#01505B) (BD-Pharmingen, La Jolla, California) as described²⁴. We also used splenocytes in a standard 4-h ⁵¹Cr-release assay to assess cytotoxicity against CT-26-FLK-1 and CT-26 target cells.

Evaluation of anti-angiogenic effects. C57BL/6J mice ($n = 8$) were injected into the sternal region with 250 μ l growth factor-reduced Matrigel (#354230, BD Biosciences, Bedford, Massachusetts) containing 400 ng/ml murine VEGF (#450-32, PeproTech, Rocky Hill, New Jersey) or bFGF (#100-188). Endothelium was stained 6 d later by i.v. injection of 200 μ l (0.1 mg/ml) *Bandiera simplicifolia* lectin I, Isolectin B4 conjugated with fluorescein (Vector Laboratories, Burlingame, California). 30 min later, mice were killed and lectin-FITC was extracted from 100 μ g per plug in 500 μ l RIPA lysis buffer, centrifuged and its content in the supernatant quantified by fluorimetry (490 nm).

In vivo depletion of CD8⁺ T cells. We depleted CD8⁺ T cells by weekly i.p. injections of 500 μ g rat anti-mouse monoclonal antibody to CD8 (RH.495) as described³². Controls included non-depleted animals either vaccinated with pcDNA3.1-FLK1 or pcDNA3.1.

Immunohistochemistry. We stained cryosections (10 μ m) fixed in paraformaldehyde. Antibody to CD31 (Pharmingen, San Diego, California) was incubated with rhodamine-conjugated secondary antibody, blocked with rat serum, followed by immunostaining with a FITC-conjugated antibody to CD8. Photomicrographs were captured with a laser scanning confocal microscope (Biorad, Hercules, California). Frozen tissue sections were stained with the Techmate Automate (Dako, Hamburg, Germany). Single stained serial sections were incubated for 30 min with biotinylated antibodies, followed by the streptavidin-peroxidase complex (DAKO) and the chromogen AEC (DAKO). Double stainings were performed as described³³.

Density of antigen-expressing cells was determined by counting of high-power fields.

Evaluation of possible side effects. To test wound healing, wounding was performed as described^{34,35}. We inflicted 4 circular wounds of 3-mm diameter each on the upper back of C57BL/6J mice ($n = 6$), 2 wk after immunization with pcDNA3.1-FLK1 or the empty vector. Time until wound closure was noted. To evaluate fertility, 2 wk after the third immunization with either pcDNA3.1-FLK1 or with empty vector, female C57BL/6J mice ($n = 9$) were allowed to cohabitate with 3 males. The days until parturition and number of pups were noted. To test neuromuscular performance, we evaluated vaccinated and control mice by both the wire hang test and the footprint test^{36,37} as well as by overall behavior and determination of body weight. To test hematopoiesis, animals were subjected to complete peripheral blood counts and differentials up to 10 mo after immunization.

Statistical analysis. The statistical significance of differential findings between experimental groups and controls was determined by Student's *t*-test and considered significant if two-tailed *P* values were <0.05.

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Competing interests statement

The authors declare that they have no competing financial interests.

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